OIPE JC42
JUL 15 2003
PATENT & TRADEMARK OFFICE

Patent Application
Docket No. USF-T147X
Serial No. 09/903,993

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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Examiner	:	Daniel M. Sullivan
Art Unit	:	1636
Applicants	:	Lars Nilsson, Huntington Potter, Gary W. Arendash
Serial No.	:	09/903,993
Filed	:	July 13, 2001
For	:	Transgenic Animal and Methods

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF

LARS NILSSON, HUNTINGTON POTTER, AND GARY W. ARENDASH

UNDER 37 C.F.R. §1.131

Sir:

DRS. LARS NILSSON, HUNTINGTON POTTER, and GARY W. ARENDASH hereby declare:

THAT, we are co-inventors of the technology described and claimed in the above-identified U.S. patent application;

THAT, we have read and understood the Office Action dated February 11, 2003 in the above-identified application, and the references cited in the Office Action; and

Being thus duly qualified, do further declare as follows:

Prior to October 24, 1999, my co-inventors and I had completed our invention of a transgenic mouse having a genome containing a first transgene encoding human α_1-antichymotrypsin (hACT) and a second transgene encoding human amyloid precursor protein (hAPP), wherein the first transgene is operably linked to a modified glial fibrillary protein (GFAP) promoter capable of driving expression of the hACT transgene within the brain of the mouse at sufficient levels to cause an increase in amyloidosis, as described in the subject application, as evidenced by the following:

1. Prior to October 24, 1999, we produced a transgenic mouse line containing a cDNA fusion-construct with a 6kbp mouse GFAP promoter and 200 bp of the 5'-end of the GFAP (Sarid, J., *J Neurosci Res.*, 28(2):217-228, Feb., 1991) attached to the human ACT cDNA clone. In addition, several ATG start codons in the GFAP part of the transcript that would likely interfere with ACT expression were deleted and the hACT gene placed downstream of the GFAP transcription start site. This is evidenced by page 3 of Exhibit A and pages 1 and 2 of Exhibit B.

2. Prior to October 24, 1999, we assayed the modified GFAP-hACT construct using Northern blot hybridization and immunoprecipitation/Western blot and confirmed the construct's ability to support hACT mRNA and protein expression after transient infection into C6 glioma cells. This is evidenced by page 3 of Exhibit A and pages 3 and 4 of Exhibit B.

1. Prior to October 24, 1999, we produced transgenic mice (FVB/N strain) using the modified GFAP/hACT expression plasmid and oocyte injection. Prior to October 24, 1999, we used polymerase chain reaction (PCR) to confirm the presence of the coding sequence of the transgene in two founder animals and to show that the transgene is passed intact to half of the progeny of these founders mated with wild-type mice. The successful expression of hACT in the brains of several heterozygous transgenic ACT mice, but not in wild-type mice, was demonstrated prior to October 24, 1999 using non-radioactive immunoprecipitation/Western blots. The major band co-migrated with ACT purified from human serum, indicating that the mice not only express human ACT, but also correctly glycosylate it. This is evidenced by page 3 of Exhibit A.

4. Prior to October 24, 1999, we mated transgenic strains of mice expressing an Alzheimer's disease mutated form of the human APP gene (PDGF-APP) with the ACT transgenic mice, producing PD APP/ACT double transgenic mice. This is evidenced by page 4 of Exhibit A and pages 3 and 4 of Exhibit B.

5. Prior to October 24, 1999, we confirmed the genotype of the PD APP/ACT double transgenic mice and, using immunocytochemistry and PCR, confirmed the expression of both the hACT and hAPP transgenes within the brains of the mice, as well as the production of amyloid-β (Aβ) peptide complexed with the hACT. This is evidenced by pages 5-8 of Exhibit B.

The above averments are evidenced by our Invention Disclosure that we submitted to the Division of Patents and Licensing at the University of South Florida (assignee of record) prior to October 24, 1999, the pertinent portions of which are submitted herewith as Exhibit A, and by our laboratory notebooks, the pertinent portions of which are submitted herewith as Exhibit B.

We hereby further declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

By: _____
Lars Nilsson, Ph.D.

_____
Date

By: _____
Huntington Potter, Ph.D.

7/11/03
Date

By: _____
Gary W. Arendash, Ph.D.

_____
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By: Lars Nilsson
Lars Nilsson, Ph.D.

July 11, 2003
Date

By: _____
Huntington Potter, Ph.D.

_____
Date

By: _____
Gary W. Arendash, Ph.D.

_____
Date

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and is not a part of the Official Record

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EXHIBIT A

Form revised [redacted]

APPENDIX 11 ✓ DEAN [redacted]

CONFIDENTIAL Copied WD [redacted]

INVENTION DISCLOSURE FORM
DIVISION OF PATENTS AND LICENSING
UNIVERSITY OF SOUTH FLORIDA
FAO 126

DATE: [redacted]

DISCLOSURE NO.: 96B046

INVENTOR SUBMITTING DISCLOSURE: Huntington Potter

TITLE: Professor and Eric Pfeiffer Chair for Research on Alzheimer's Disease

BUSINESS ADDRESS: MDC 7 (Biochemistry) 12901 Bruce B. Downs Blvd

E-MAIL ADDRESS: hpotter@hsc.usf.edu

PHONE NUMBER: 974-5369

SIGNATURE: [signature]

TITLE OF INVENTION: ~~Antichymotrypsin~~ Transgenic mice expressing human antichymotrypsin in the brain

1. DIRECTIONS:

This form is to be completed and submitted to the Division of Patents and Licensing by any Researcher who believes he or she has developed a new invention. The purpose of this form is to permit the Divison of Patents and Licensing to determine whether any legal protection for the invention will be sought. **HENCE IT IS IMPORTANT THAT ALL QUESTIONS BE ANSWERED AS ACCURATELY AS POSSIBLE.**

2. THE INVENTION

A. What is the problem this invention addresses?

Antichymotrypsin is an inflammatory human protein that plays an important role in the pathogenesis of Alzheimer's Disease. The invention addresses the need for a mouse model of Alzheimer's disease that includes the expression in the brain of antichymotrypsin from humans.

B. In the space provided, please briefly describe and explain your invention in the form of an abstract. If the space provided is not sufficient, kindly attach the abstract to this Disclosure Form.

See attached

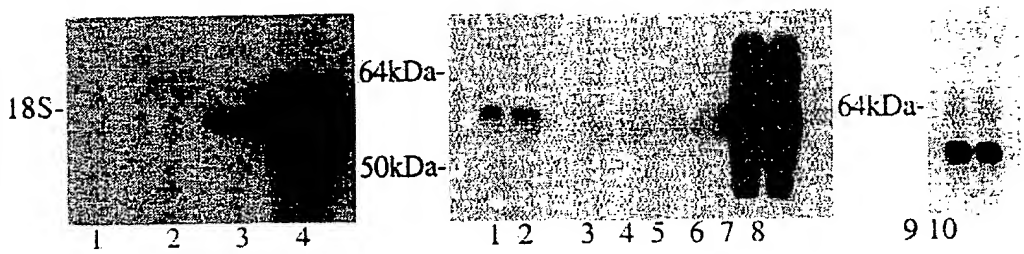
ABSTRACT

Invention Disclosure by Lars Nilsson and Huntington Potter

Biochemical, genetic, and epidemiological evidence indicates that inflammation is an essential part of the pathogenesis of Alzheimer's disease. For example, we have learned, from both in vivo and in vitro experiments in our and other labs, that several acute phase/inflammatory molecules in the brain, specifically antichymotrypsin (ACT) and apolipoprotein E (apoE) can promote the formation of the neurotoxic amyloid deposits that are the main pathological hallmark of the disease. They do this by binding directly to the Aβ peptide and promoting its polymerization into amyloid filaments. Furthermore, there is a massive overproduction of ACT in affected areas of the Alzheimer brain that is evidently caused by activation of ACT mRNA synthesis in astrocytes by the inflammatory cytokine IL-1 released from activated microglia. In order to develop a mouse model of the inflammatory aspect of Alzheimer's disease, we have created a transgenic mouse line that expresses human ACT in astrocytes. This mouse line will also be mated to various other lines over-expressing the Alzheimer amyloid precursor protein and having zero, one, or two copies of the mouse or human apolipoprotein E gene to generate additional novel lines. These various lines will be used to determine whether, as has recently been shown for apoE, ACT is an amyloid promoting factor in vivo, either alone, or together with apoE and/or over-expression of APP. The mice will also serve as targets for testing potential anti-amyloid and anti-inflammatory drugs for use in Alzheimer's disease therapy.

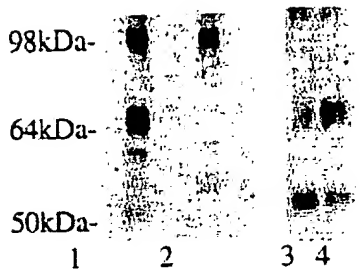
The second attempt to generate ACT mice was performed by Carmela Abraham, after she graduated from our lab, in collaboration with Leonard Mucke. In this case, the GFAP promoter was used, as we had earlier discussed in my lab. However, no expression occurred in the brains of the animals, even after stab wound was used in induce gliosis (C. Abraham, personal communication). Recently, we decided to try again with the GFAP promoter modified in such a way as to be more likely to drive expression from a fusion mRNA. Appreciation that the mRNA start site in GFAP was more upstream than previously thought, and the consequent removal by site-directed mutagenesis of several potentially confounding ATG codons in the 5'UTR of GFAP greatly increased the levels of ACT mRNA and protein expression in transfected glioblastoma cells. Specifically, we have generated a transgenic mouse line containing a cDNA fusion-construct with a 6kbp mouse glial fibrillary acidic protein (GFAP) promoter and 200bp of the 5'-end of the GFAP (Sarid, 1991) attached to the human ACT cDNA clone. In addition, several ATG start codons in the GFAP part of the transcript that previously interfered with ACT expression have been deleted and the human ACT gene placed downstream of the GFAP transcription start site. The non-coding 3'UTR of the mRNA is derived from the rat preproinsulin II gene, which provides a 3' intronic region and a polyadenylation (polyA) site.

As a first test of function, the GFAP-ACT construct was assayed for its ability to support ACT mRNA and protein expression after transient transfection into C6 glioma cells. This cell-line has been used since it is of rat origin and allows the human ACT mRNA and protein to be easily distinguished from any rat species. The results are shown below.



Left: *Northern blot hybridization with an ACT probe of polyA+ mRNA from (lane 1) untransfected and (lane 2) GFAP-hACT DNA , and (lane 3) untreated and (lane 4) IL-1 - treated U373 MG human astrocytoma cells showing the position of the native human ACT transcript (which is slightly smaller than the fusion gene transcript).*
Right Panels: *Immunoprecipitation/Western blot showing ACT protein in (lane 1-2) transfected, (lanes 3-4) untransfected C6 glioma cells. UntransfectedUntransfected cell spiked with 10pg and 1ng respectively of human ACT are shown in lanes 5, 6. Lanes 7 and 8 (and a shorter exposure of lanes 9 and 10) show cells transfected with a CMV-ACT construct which expresses ACT at high levels.*

Transgenic mice (FVB/N strain) were then generated using the GFAP/ACT expression plamid and conventional oocyte injection. PCR has been used to confirm the presence of the complete transgene in three founder animals and to show that the transgene is passed intact to half of the progeny of these founders mated with wild type mice. Some of the heterozygous offspring have already been inbred to generate homozygous transgenic animals. The successful expression of human ACT in the brains of several heterozygous transgenic ACT mice, but not in wild type mice, was demonstrated using non-radioactive Immunoprecipitation/Western blots. The major band comigrated with ACT purified from human serum, indicating that the mice not only express human ACT, but also correctly glycosylate it.



Expression of human ACT in transgenic (lane1) but not in normal (lane 2) mice. Lane 3 and 4 show 100pg and 1ng respectively of purified human ACT exposed for ten times longer than lanes 1 and 2.

The ACT mice may, by themselves develop Alzheimer-like pathology such as amyloid deposits, nurofibrillary tangles, synapse loss, and neuronal degeneration and may develop behavioral and memory deficits. We will also mate the human ACT transgenic mice with transgenic strains that express an Alzheimer's disease mutated form of the human APP gene (PDGF-APP), and which therefore produce numerous congophilic plagues in the hippocampus and cortex. The additional presence of an expressed ACT gene in the progeny of this cross is expected to increase the rate or extent of amyloid formation and of the development of other Alzheimer-like pathology.

Recent results of mating the PDGF-APP mice to apoE knockout mice have indicated that apoE is essential for amyloid formation (Bales et al, 1997). These APP/apoE KO mice will also be mated to the ACT transgenics to determine whether and how ACT and apoE interact to promote amyloid formation. For example, in the APP+/+ apoE-/- mice, no amyloid develops up to two years of age. If ACT expression is introduced into this background, amyloid should now form. One or two copies of apo E may contibute to an optimal amyloid promoting effect. The various strains will also be analyzed for relative behavior changes.

The claims should cover not only the ACT mice, but any progeny of mating the ACT mice to other mice such that the progeny express human ACT in the brain. The specific mice that will be important for such matings are indicated in the text above.

EXHIBIT B

Genomic DNA

GFAPACT/ACT/AflIII

#8782
#8783
#8784
#8785
#8786
#8788
#8789
#8790
#8791 (1kb)
no kinase

GFAPACT/GFAP3FND

GFAPACT/ACT/AflIII GFAPACT/GFAP3FND

#8782
#8783
#8784
#8785
#8786
#8788
#8789
#8790
#8791 (4kb)
no kinase

#8782
#8783
#8784
#8790

} clones possible for GFAPACT + ACT/AflIII

[redacted]

Stat3insulin/polyA3END

#8782
#8783
#8784
#8785
#8787
#8788
#8789
#5790
#8791
construct-pos. ctrl.
(GFAP)
neg. control

#8783
#5754
#8790
} scores positive for Stat3insulin + polyA3END

GFAPACT/Ecoinsulin

#8782
#8783
#8784
#8785
#8787
#8788
#8789
#5790
#8791
construct pos. ctrl.
(GFAP)
neg. control

IP3END

all scores negative

GFAPACT/ACTHUM3

#8782
#8783
#8784
#8785
#8787
#8788
#8789
#5790
#8791
construct pos. ctrl.
(GFAP)
neg. control

#8754 scores positive for GFAPACT/ACTHUM3

ACT - Aβ - complex IP-Western Aβ

[redacted]

1. APP -/- , ACT +/- => Bluemarked, #1, ♀, 3 months
2. APP -/- , ACT -/- => — " — , #2, ♀, 3 months
3. APP -/- , ACT +/- => (#28/wt), #17, ♀, 2,5 months
4. ~~25~~ ng ~~ACT~~ ACT, serum
5. 50 ng ACT - serum

1 ml

STEN-lysis: (10 ml)

2x STEN	5 ml
NP-40	80 µl
PMSF	25 µl
PI-cocktail	100 µl
H_2O	4,6 ml
	9,8 ml

Aβ-standards

1 µl + 12 µl => load all => ? ng
1/2 µl + 12 µl => load all => 5 ng
1/2 µl + ? µl => load 12 µl => 2 ng
1/2 µl + 200 µl => load 12 µl => 500 pg

Add BSA after removal and centrifugation! + BSA (1 µg/µl) + 200 µl => 40 µl/2 ml

→ rotor had been standing overnight, + 3hr rotation!

[redacted]

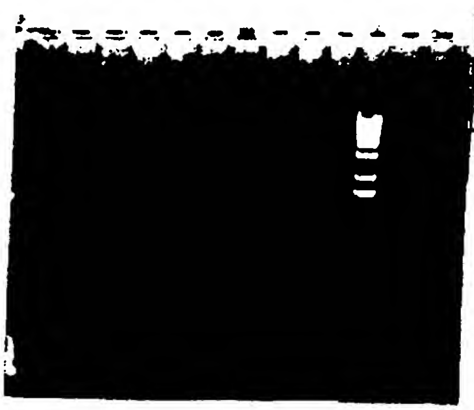
Wash: – Tris + Triton + NaCl (6x 10 min)
– TNT (3x 10 min)
– ? (30 µl, + 95°C, 10 min) with DTT
– ? + loading

loading: 10 µl ACT Aβ (detect ACT-ab) - 8% gel
10 µl ACT - Aβ (detect Aβ-ab) - 8% gel
10 µl Aβ (detect Aβ-ab) - 15% gel

? — 21 (#1 - #7 ; 1 µl)
— #1, #2, #17 ; 1 µl)
negative control (2 µl)

Mastermix: GFAPACT – ? µl
(13) ACTA ? – ? µl
RC ? – ? µl
$MgCl_2$ – 15 µl
dNTP – 3 µl
Taq-pol – ? µl
H_2O – 130 µl
234 µl : ?

block 2
– 56°C, annealing
– 27 cycles
– 72°C, 1 min



(21)
APP+ ACT+ APP- ACT-
#1 - #7, #1, #2, #17, ? neg (2 µl)

[redacted]

Aβ - IP

IP - down 66ng (4µl) add 0,5ml STEN-lysis
17ng (1µl) add 0,5ml —"—
5ng (½µl) add 0,5ml —"—

1µl $Aβ_{1-40}$-antibody add 1,5ml (dil 1:1500) for IP
Western: → 4G8-antibody (primary 1:2500)
→ Secondary (1:5000)

[redacted]

Southern Hot - APP

Blue-marked #1, #2, #3
→ fetal extract 50µg
→ cut ~20µg with BglII/HindIII, NEB2
↳ ⓐ. fetal extr.
ⓑ. Qiagen

[redacted]
→ cut ~2µg with BglII ; 2µg HindIII, 2µg nothing
as a prelim. test

	Qiagen	Phenol
①	0,5µg/µl	1,5µg/µl
②	0,9µg/µl	1,9µg/µl
③	0,75µg/µl	1,0µg/µl

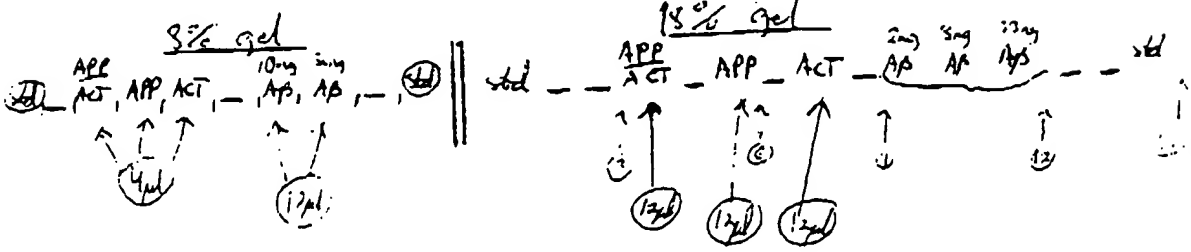
ACT - Aβ - complex Western-IP

(1). APP$^{+/-}$, ACT$^{+/-}$, Blue marked #5, ♀
(2). APP$^{+/-}$, ACT$^{-/-}$, — " — #3, ♀
(3) APP$^{-/-}$, ACT$^{+/-}$, #28/wt — #76, ♀
(4). 10ng ACT-serum
(5) 30ng ACT-serum

STEN-lysis: 2x STEN 6ml
NP-40 96µl
PMSF 30µl
PI cocktail 120µl
H_2O 5,51ml
11,76ml

Add BSA after homogenization: + BSA (100mg/ml) + 240µl ⇒ 20µl/1ml

Wash:
- TNE + triton (6x 10min)
- TNE/EDTA (2x 10min)
- Sample buffer (16µl, +85°C, 10min) - with DTT
- dye + loading



[redacted]

ACT - Immunohistochemistry

10st
- 2 sections from #4, #6, #8 and #9
- 2 sections without prim ACT-Ab. (#6 + #9)

4 positive control
- 2 sections with prim ACT-Ab. (#3 and #14) – ACT+
- 2 sections without — " — (#3 and #14)

Prim: ACT-pAb, Accurate, 1:400, no block
Sec: anti-rabbit, 1:300, no block
ABC: 50% block
Detection: DAB

[redacted]

DAB 10
- 1 swith from #3, #14, #18 (ACT-1H)
- 2 swith neg control
- 1 swith from #3, #14, #18 (GFAP-1H)
- 2 swith neg control

SG 5
- 1 swith from #3, #14, #18 (GFAP-1H)
- 2 neg control

ACT-DAB

Prim: ACT-pAb, 1:400, no block
Sec: anti-rabbit, 1:300, no block
ABC: 50% block
Detection: DAB

GFAP

Prim: 1:400, no block
Sec: 1:300, no block
ABC: ~~50%~~ 50% block
Det: DAB and SG

APP - IH

Prim: 1:10, Boehringer 22C11
Sec: 1:300, anti-mouse, no block
ABC: 20% block

R1280

Prim: 1:1000 or 1:3000
Sec: 1:300, anti-rabbit
ABC: 20% block

4G8

Prim: 1:1000 or 1:3000
Sec: 1:300, anti-mouse
ABC: 20% block

GFAP - IH

Prim: 1:400, Sigma
Sec: 1:300, anti-mouse, no block
ABC: 20% block

Aβ - IH (R1280)

Prim: 1:5000 R1280, no block (1:6250 dilution extra with 20% block)
Sec: 1:300, anti-rabbit, no block
ABC: 20% block

- purple (#59 - #89) - ? 1st
- neg control

Block 2
- 50°C annealing
- 27 cycles
- 72°C, 7min

Mastermix: (35)

GFAPAC72	70µl
ACTAAIII	70µl
PCR-buff	70µl
$MgCl_2$	42µl
dNTP	5µl
Taq-pol.	4µl
H_2O	366µl
	630µl

59-68, 69-77

73-77 IH neg (3)

(17)

[redacted] – purple (#57-#89)
– neg. control

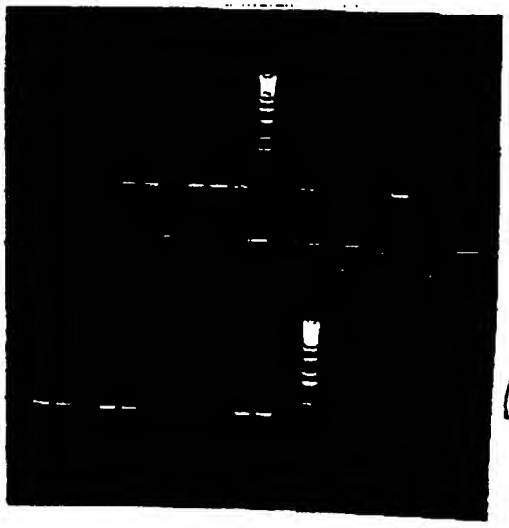
Block 2
- 56°C Annealing
– 30 cycles
– 72°C, 3 min

Mastermix: (35)

ApoE5	70 µl	2 µl
ApoE3b	70 µl	2.2 µl
PCR buff.	70 µl	2.2 µl
$MgCl_2$	42 µl	1.3 µl
dNTP	8 µl	2.5 µl
Taq-pol	4.2 µl	1.3 µl
H_2O	366 µl	11.5 µl
	630 µl	19.8 µl

#59-#68, std, #69-#77

ApoE5
ApoE3b



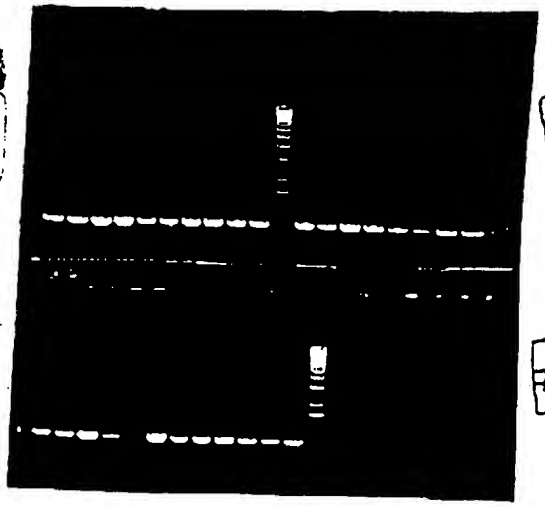
Mastermix: (35)

NEOApoE	70 µl
ApoE3b	70 µl
PCR buff.	70 µl
$MgCl_2$	42 µl
dNTP	8 µl
Taq-pol.	4.2 µl
H_2O	366 µl
	630 µl

#78-#89, std, neg (3#)

Melinda (#59-#66)

NEOApoE
ApoE3b



59-68, std, 69-77

78-89, std, neg (3#)